International Journal of Microbiological Research 1 (2): 50-61, 2010
ISSN 2079-2093

Influence of Probiotics Mixture on *Salmonella typhimurium* in Mice

[1]*J. El-Jakee,* [2]*I.M. Moussa,* [3]*S.A. Nada,*
[1]*Kh. F. Mohamed,* [4]*M.H. Ashgan and* [5]*M.L. Mohamed*

[1]Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt
[2]Center of Excellence in Biotechnology, King Saud University,
P.O. Box 2460 Riyadh, Kingdom of Saudi Arabia
[3]Pharmacology Department, National Research Centre, Giza, Egypt
[4]College of Applied Studies and Community Service, King Saud University,
P.O. Box 2460 Riyadh, Kingdom of Saudi Arabia
[5]VACSERA, Egypt, 51 Wezaret El-Zeraa-Agouza-Giza-Egypt

Abstract: The influences of the following four different types of probiotics were studied on *Salmonella typhimurium*: Mixture of *Lactobacillus acidophilus*, *Bifidobacterium bifidus* and *Streptococcus thermophilus* strains (ABT-3), *Lactobacillus acidophilus* strain (La-5), *Bifidobacterium bifidus* strain (Bb-12) and *Lactobacillus helveticus* strain(Lh-02). Firstly, the antagonistic activities of the used strains against *Salmonella typhimurium* were studied *in vitro*. Comparative studies have been conducted to investigate some of the influences of these probiotics on *Salmonella typhimurium* infection in mice. The study included the effect of each probiotic on mice body weight, *Salmonella typhimurium* colony count in feces, secretary IgA titer in intestinal washing, lysozymal activity in serum, effect on serum biochemical parameters as AST, ALT, creatinine and uric acid, as well as their antioxidant activities against *Salmonella typhimurium* by SOD. It could be concluded that the mixed culture of probiotic strains could increase the protective and treatment effects against *Salmonella typhimurium* infection and that they are more effective than using the individual probiotic strain.

Key words: Probiotics · *S. typhimurium* · IgA · AST · ALT · SOD

INTRODUCTION

Salmonella is an important pathogen to the food industry and has been frequently identified as the etiological agent of foodborne outbreaks [1]. *Salmonella enterica* serovar Typhimurium is among the most common *Salmonella* serovars causing salmonellosis in the United States.

Annually, it accounts for more than 40,000 reported cases, 500 deaths and considerable financial costs that are in excess of $50 million [2].

Probiotics have been defined by The Food Agricultural Organization/World Health Organization (FAO/WHO) as "live microorganisms which when administered in adequate amounts confer a health benefit to the host" [3]. Probiotics are nonpathogenic microorganisms that have a positive influence on the health or physiology of the host. Lactobacilli have the longest history as probiotics and pertain to the predominant gastrointestinal microbiota of laboratory and farm animals [4]. However, bifidobacteria colonize the human neonatal intestine soon after birth and inhabit the gastrointestinal tract throughout life [5]. Parkes *et al.* [6] established an etiological framework for the use of probiotics in irritable bowel syndrome (IBS) in both primary and secondary care.

This work was carried out to study the effect of commercial probiotic combination (mixture of *Lactobacillus acidophilus*, *Bifidobacterium bifidus* and *Streptococcus thermophilus* - ABT-3) in comparison to individual probiotic strains of *Lactobacillus acidophilus* (La-5), *Bifidobacterium bifidus* (Bb-12) and *Lactobacillus helveticus* (Lh-02) on *Salmonella* Typhimurium.

Corresponding Author: Khaled F. Mohamed, Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt, E-mail: kelamry@daralfarouk.com.eg.

MATERIALS AND METHODS

Strains: *Salmonella typhimurium* ATCC 14028; ABT-3: mixed culture containing *Lactobacillus acidophilus*, *Bifidobacterium bifidus* and *Streptococcus thermophilus* strains; La-5: *Lactobacillus acidophilus* strain; Bb-12: *Bifidobacterium bifidus* strain; and Lh-02: *Lactobacillus helveticus* strain. All strains were in freeze-dried form from Christian Hansen Laboratory, Horsholm, Denmark.

***In Vitro* Antagonistic Activity of the Probiotic Supernatants Against *Salmonella typhimurium* [7]:** De Man, Rogosa and Sharpe broth (MRS broth) from Oxoid Ltd., Basingstoke, UK, were inoculated with 0.1 g of probiotic, incubated at 42°C for 72 hours [8] and centrifuged to obtain the supernatant. By a sterile swab, *Salmonella typhimurium* with a concentration of 1/2 McFarland (1.5 X 10^8 CFU/ml) was spread over a nutrient agar plate and incubated at 37°C for 24 hours. 10 µl of each supernatant of each probiotic incubated in MRS broth was inoculated in wells in the nutrient agar and incubated at 37°C for 24 hours. The inhibition zones were measured.

Preparation of Probiotic Milk: Probiotic milk was prepared from 9.5% reconstituted skimmed milk (SM) [9]. Only 4 types of probiotic milk were prepared, each 0.1 ml containing 10^7 CFU of the used probiotics.

Experimental Design: The experiments were carried out on a total of 90 white albino 6-week-old male mice, obtained from the Animal House Colony, National Research Center (Giza, Egypt). They were divided into two main experiments: protection and treatment experiments.

Four groups were used for the protection experiment: two for the treatment experiment and three as control groups.

Protection Experiment: A dose of 0.1 ml containing 10^7 CFU probiotic in SM was administrated to the correspondence group by gavage. SM containing ABT-3 was administrated to group 1 (G1). SM containing La-5 was administrated to group 2 (G2). SM containing Bb-12 was administrated to group 3 (G3). SM containing Lh-02 was administrated to group 4 (G4). The same dose was administrated daily to the animals for 6 successive days before the challenge. A control group (group 5 or G5) was administrated with 9.5% reconstituted SM according to the same schedule of the corresponding experimental groups. Then, each mouse was orally challenged with 0.1 ml of the prepared *Salmonella typhimurium* suspension (1.5 X 10^8 CFU/ml) [10]. The same dose of each probiotic milk was repeated daily to the animals for 9 days after the challenge (booster dose). Group 5 was administrated with 9.5% reconstituted SM according to the same schedule. The feces of the mice in each group were individually collected after the administration of the sixth dose of SM and on the second, fifth and ninth days post infection for the detection of *Salmonella typhimurium*.

Treatment Experiment: Each mouse was orally infected with a single 0.2 ml dose of the prepared *Salmonella typhimurium* suspension (1.5 X 10^8 CFU/ml). Post to oral *Salmonella* infection, 0.2 ml containing 10^7 CFU/ml^{-1} of probiotic milk was administrated to the correspondence group by gavage. SM containing ABT-3 was administrated to group 6 (G6). SM containing La-5 was administrated to group 7 (G7). The treatment was administrated daily for 9 days. The control group (group 8 or G8) was treated with 9.5% reconstituted SM according to the same schedule of the corresponding experimental groups. The feces of the mice at each group were individually collected on the first, second and third days post infection for the detection of *Salmonella typhimurium*.

***Salmonella typhimurium* Colony Count in Feces of Mice:** One gram of feces was freshly collected from each group separately; feces were weighed and diluted in regenerated sterile buffered saline (pH 7.2). Viable *Salmonella typhimurium* organisms were determined [9].

DETECTION OF THE EFFECT OF PROBIOTIC ON SOME IMMUNOLOGICAL AND SERUM BIOCHEMICAL PARAMETERS OF MICE

IgA Titer in Intestinal Wash: The mice intestinal wash [11]) was examined for IgA titer using ELISA [12]) on the first, second and ninth days after oral challenge with *Salmonella typhimurium* among the protective groups and on the third and tenth days after challenged among the treated groups.

Lysozyme Assay: The lysozyme concentration in the serum of mice was assayed according to Schultz [13] on the second and ninth days after oral challenge with *Salmonella typhimurium* among the protective groups and on the tenth and fifteenth days among the treated groups.

ASPARTATE AMINOTRANSFERASE (AST), ALANINE AMINOTRANSFERASE (ALT), URIC ACID and CREATININE

At the end of the experimental time, blood samples were collected from retro-orbital venus plexus in sterile test tubes and centrifuged for serum separation at 1500 rpm for 15 min to estimate AST, ALT—according to the assay described by Kaplan and Pesce [14] —uric acid and creatinine according to the assay described by Tietz *et al*. [15].

Superoxide Dismutase (SOD): The measurement of SOD in serum of mice was assessed according to the assay described by Podezasy and Wei [16] at the end of the experimental time.

Statistical Analysis: The statistical procedures used were according to Snedecor [17]. The student t-test was used in addition to the Analysis of Variance Fisher (F-test).

RESULTS AND DISCUSSION

Successful probiotic bacteria are usually able to colonize the intestine, at least temporarily, by adhering to the intestinal mucosa. Adhesion of probiotic microorganisms to the intestinal mucosa is considered important for many of the observed probiotic health effects, such as antagonistic activity against enteropathogens, modulation of immune system [18] and increased healing of damaged gastric mucosa [19].

Understanding how probiotics exert their beneficial effects is the issue of debate nowadays. Four mechanisms have been summarized to explain the protective effects of probiotics: antagonism through the production of antimicrobial substances [20], competition with the pathogen for adhesion sites or nutritional sources [21], immunomodulation of the host [22] and inhibition of the production of bacterial toxins [23]. The first three mechanisms are ordinarily attributed to lactic acid bacteria, while the last two are more specifically attributed to yeast [24].

The antagonistic activity of the used probiotics (ABT-3, La-5, Bb-12 and Lh-02) against *Salmonella typhimurium in vitro* is shown in Table 1 and Figure 1. It is clear that the used probiotics had inhibitory effect on *Salmonella typhimurium* and that the zone of inhibition ranged from 8 to 11 mm. It has previously been reported that *Lactobacillus* strains inhibit the growth of Gram-negative pathogenic bacteria [20]. This growth-inhibiting activity has generally been attributed to the fact that *Lactobacillus* species lower the pH and/or produce lactic acid. For example, strains of *L. acidophilus*, *L. casei* subsp. *rhamnosus* and *Lactobacillus bulgaricus* inhibited the growth of clinical isolates of *H. pylori* [25,26], while *L. casei* subsp. *rhamnosus* strain Lcr35 reduced the growth of enteropathogenic *Escherichia coli*, enterotoxigenic *E. coli* and *Klebsiella pneumoniae* [27]. The data reported by Fayol-Messaoudi *et al.* [28] showed that *Lactobacillus* strains induce complete inhibition of the growth of serovar Typhimurium SL1344 that results mainly from the effect of an acid pH.

In this study, the effects of probiotics on the infection dynamics of *Salmonella typhimurium*, body weight, *Salmonella typhimurium* colony count in feces, humeral immune response and some biochemical parameters in mice were investigated. Probiotics had no significant effect on body weight, as shown in Tables 2 & 3 and Figures 2 & 3. Conflicting reports were recorded regarding the effect of probiotics supplementation on average daily gain; some showed improvement on body weight gain in calves and cattle by 6-24 % [29 - 32], while other reports stated that supplementation in calves and cattle had no effect on body weight [33,34]. The beneficial effect of probiotic was thought to result, in part, from improved intestinal function.

Tables 4 & 5 and Figures 4 & 5 illustrated that the level of the viable *Salmonella typhimurium* was lower in the protected and treated groups of mice than in the control groups. The difference was significant among the mice protected or treated with ABT-3 (G 1 & 6) and La-5 (G 2 & 7). Silva *et al*. [10] observed improved survival for mice pretreated with *Bifidobacterium longum* during challenge with *Salmonella* spp., but without affecting

Table 1: Results of antagonistic activities of probiotics against *Salmonella typhimurium in vitro*

Probiotic	*Measurement of inhibition zone*
ABT-3	10 mm
La-5	11 mm
Bb-12	8 mm
Lh-02	11 mm

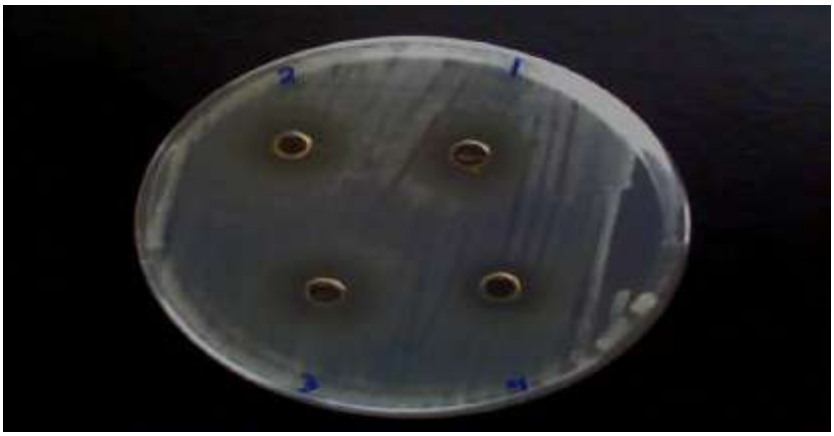

Fig. 1: Antagonistic activities of probiotics against *Salmonella* Typhimurium *in vitro*
Zone 1 shows the antagonistic activity of probiotic ABT-3
Zone 2 shows the antagonistic activity of probiotic La-5
Zone 3 shows the antagonistic activity of probiotic Bb-12
Zone 4 shows the antagonistic activity of probiotic Lh-02

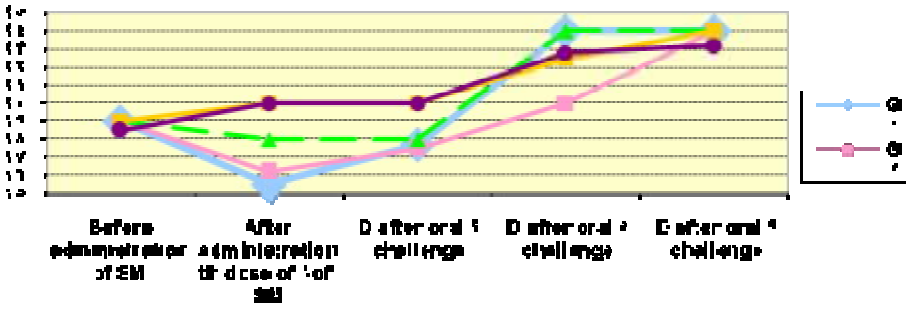

Fig. 2: The body weight of the examined mice groups in the protective experiment
* D = Day
* SM = Skimmed milk
G1 was administrated with SM supplemented with probiotic ABT -3
G 2 was administrated with SM supplemented with probiotic La-5
G 3 was administrated with SM supplemented with probiotic Bb-12
G 4 was administrated with SM supplemented with probiotic Lh-02
G 5 was administrated with SM only.

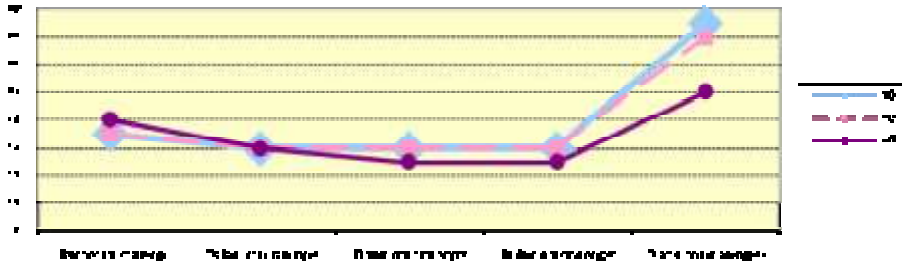

Fig. 3: The body weight of the examined mice groups in the treatment experiment
* D = Day
* SM = Skimmed milk
G 6 was administrated with SM supplemented with probiotic ABT -3
G 7 was administrated with SM supplemented with probiotic La-5
G 8 was administrated with SM only

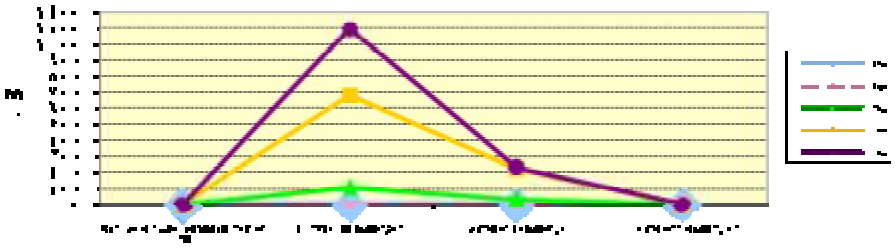

Fig. 4: Results of *Salmonella* Typhimurium colony count from fecal samples of mice in the protective experiment
* SM = Skimmed milk
G1 was administrated with SM supplemented with probiotic ABT -3
G 2 was administrated with SM supplemented with probiotic La-5
G 3 was administrated with SM supplemented with probiotic Bb-12
G 4 was administrated with SM supplemented with probiotic Lh-02
G 5 was administrated with SM only

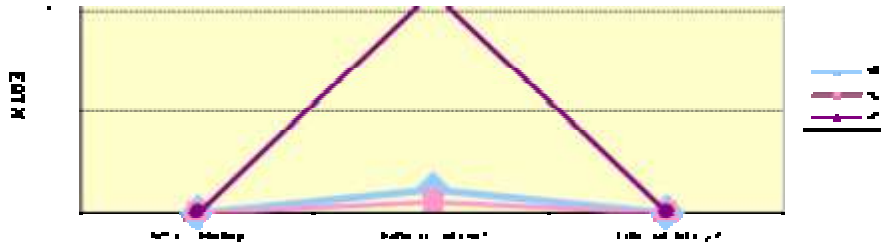

Fig. 5: Results of *Salmonella* Typhimurium colony count from fecal samples of mice in the treatment experiment
* D = Day
* SM = Skimmed milk
G 6 was administrated with SM supplemented with probiotic ABT -3
G 7 was administrated with SM supplemented with probiotic La-5
G 8 was administrated with SM only

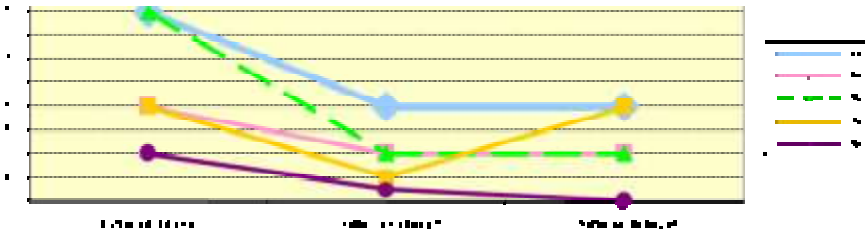

Fig. 6: Results of estimation of secretory IgA titer from intestinal washing of mice in the protective experiment
* D = Day
* SM = Skimmed milk
G1 was administrated with SM supplemented with probiotic ABT -3
G 2 was administrated with SM supplemented with probiotic La-5
G 3 was administrated with SM supplemented with probiotic Bb-12
G 4 was administrated with SM supplemented with probiotic Lh-02
G 5 was administrated with SM only

Table 2: The body weight of the examined mice groups in the protective experiment

Body Weight/g					
Groups	Before administration of SM	After administration of 6th dose of SM	Day 2 after oral challenge	Day 5 after oral challenge	Day 9 after oral challenge
1	19.0	15.5	17.7	24.0	24.0
2	19.0	16.2	17.5	20.0	24.0
3	19.0	18.0	18.0	24.0	24.0
4	19.0	20.0	20.0	22.5	24.0
5	18.5	20.0	20.0	22.8	23.2

Table 3: The body weight of the examined mice groups in the treatment experiment

Body Weight/g					
Groups	Before oral challenge	Day 1 after oral challenge	Day 2 after oral challenge	Day 3 after oral challenge	Day 10 after oral challenge
6	18.5	18	18.0	18.0	22.5
7	18.5	18	18.0	18.0	22.0
8	19.0	18	17.5	17.5	20.0

Table 4: Results of *Salmonella typhimurium* colony count from fecal samples of mice in the protective experiment

CFU/ml				
Groups	After administration of 6th dose of SM	Day 2 after oral challenge	Day 5 after oral challenge	Day 9 after oral challenge
1	0	0	0	0
2	0	0	0	0
3	0	$11x10^4$	$34x10^3$	0
4	0	$68x10^4$	$22x10^4$	0
5	0	$11x10^5$	$23x10^4$	0

* D = Day

Table 5: Results of *Salmonella typhimurium* colony count from fecal samples of mice in the treatment experiment

CFU/ ml			
Groups	Before oral challenge	Day 2 after oral challenge	Day 3 after oral challenge
6	0	$23x10^3$	0
7	0	$11x10^3$	0
8	0	$22x10^4$	0

Table 6: Results of estimation of secretory IgA titer from intestinal washing of mice in the protective experiment

Titer of IgA			
Groups	Day 1 after oral challenge	Day 2 after oral challenge	Day 9 after oral challenge
1	160	80	80
2	80	40	40
3	160	40	40
4	80	20	80
5	40	10	0

numbers of the pathogen. They postulated that this may be due to a reduced inflammatory response mediated by the probiotic treatment, but not population antagonism. Probiotic bacteria have well-established beneficial effects in the management of diarrheal diseases [35]. The data presented by Casey *et al.* [36] showed that the probiotic mixtures used led to an amelioration of diarrhea in *S.* enterica serovar Typhimurium-infected mice early in the course of infection and reduced pathogen counts over a longer time frame. This demonstrates the validity of using commercial LAB strains in the prevention of gastrointestinal infection and underlines the usefulness of the *in vitro* and *in vivo* procedures used to isolate and select the bacteria [37].

Table 6 and Figure 6 clarify that in the protective experiment, high level of IgA titer (160) was observed among mice one day after oral challenge and after the seventh dose of SM supplemented with ABT-3 (G 1) and Bb-12 (G 2) compared with the IgA titer (40) of the mice in the control group (G 5). Rautava *et al.* [38] recorded that the numbers of cow's milk-specific IgA secreting cells were significantly higher in infants receiving probiotics (*Lactobacillus* GG and *Bifidobacterium lactis* Bb-12), compared with those receiving placebo. They hypothesized that specific probiotics might promote mucosal immunological maturation in formula-fed infants. Table 7 and Figure 7 clarify that the mice treated with ABT-3 (G 6) and La-5 (G 7) had significant IgA titer (80)

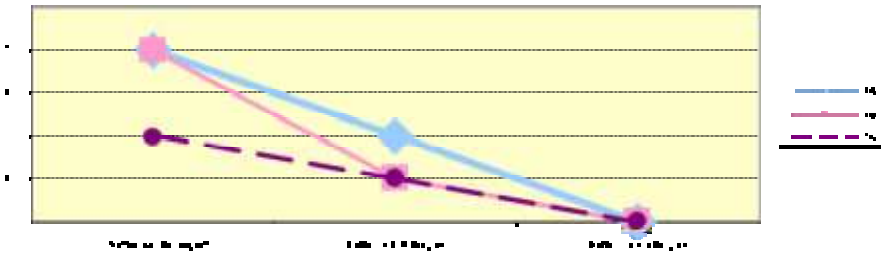

Fig. 7: Results of estimation of secretory IgA titer from intestinal washing of mice in the treatment experiment
* D = Day
* SM = Skimmed milk
G 6 was administrated with SM supplemented with probiotic ABT -3
G 7 was administrated with SM supplemented with probiotic La-5
G 8 was administrated with SM only

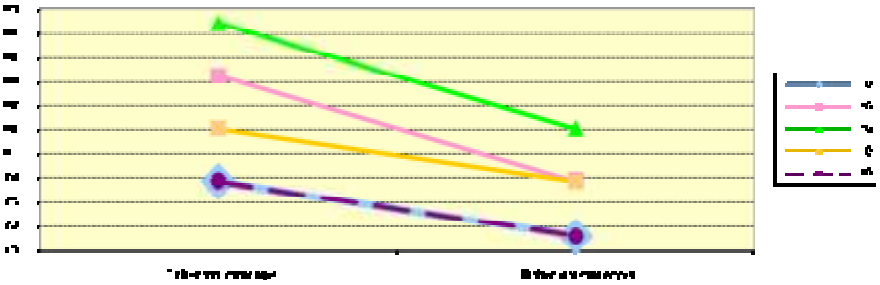

Fig. 8: Results of detection lysozymal activity of probiotic in the serum of mice in the protective experiment
* D = Day
* SM = Skimmed milk
G1 was administrated with SM supplemented with probiotic ABT -3
G 2 was administrated with SM supplemented with probiotic La-5
G 3 was administrated with SM supplemented with probiotic Bb-12
G 4 was administrated with SM supplemented with probiotic Lh-02
G 5 was administrated with SM only.

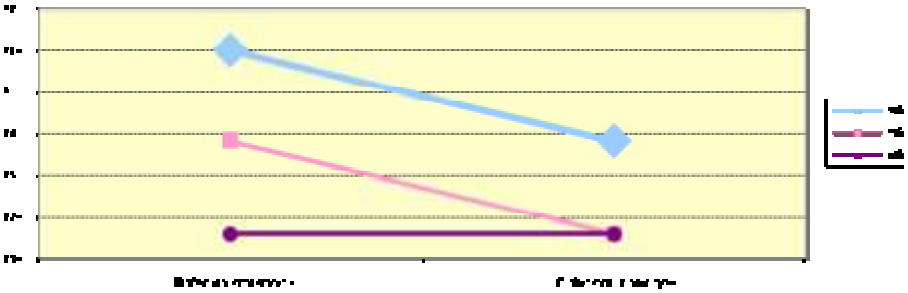

Fig. 9: Results of detection of lysozymal activity of probiotic in the serum of mice in the treatment experiment
* D = Day
* SM = Skimmed milk
G 6 was administrated with SM supplemented with probiotic ABT -3
G 7 was administrated with SM supplemented with probiotic La-5
G 8 was administrated with SM only

Table 7: Results of estimation of secretory IgA titer from intestinal washing of mice in the treatment experiment

Titer of IgA			
Groups	Day 3 after oral challenge	Day 10 after oral challenge	Day 15 after oral challenge
6	80	40	0
7	80	20	0
8	40	20	0

Table 8: Results of detection of lysozymal activity of probiotic in the serum of mice in the protective experiment

Concentration of lysozyme (µg/ml)		
Groups	Day 2 after oral challenge	Day 9 after oral challenge
1	288.30	266.23
2	332.43	288.30
3	354.50	310.36
4	310.36	288.30
5	288.30	266.23

Table 9: Results of detection of lysozymal activity of probiotic in the serum of mice in the treatment experiment

Concentration of lysozyme (µg/ml)		
Groups	Day 10 after oral challenge	Day 15 after oral challenge
6	310.36	288.30
7	288.30	266.23
8	266.23	266.23

Table 10: Results of AST, ALT, uric acid, creatinine, and SOD in the serum of mice in the protective and treated experiments

Groups/Parameters		AST Mean (µg/l)	ALT Mean (µg/l)	Uric acid (mg/dl)	Creatinine (mg/dl)	SOD U/ml
Control negative		37.67	25.17	2.70	0.42	30.83
*Protective groups	G1	39.50	29.17	2.09	0.44	21.50
	G2	43.50	27.50	2.10	0.47	22.33
	G3	39.00	28.33	2.42	0.52	26.33
	G4	38.83	26.83	2.15	0.44	24.67
	G5	48.83	35.17	3.83	0.64	19.70
**Treated groups	G6	40.33	28.67	2.55	0.46	25.67
	G7	42.67	26.67	2.30	0.44	27.17
	G8	48.83	35.17	3.83	0.64	19.70

* 9 days after oral challenge and 15th dose of SM

** 15 days after oral challenge & 9th dose of SM

three days after oral challenge and after the second dose of the treatment higher than the level of IgA titer (40) in the mice of the control group (G 8). Specific immune stimulation by probiotics through processes involving dendritic cells might be beneficial to the host immunological status and helps prevent pathogen translocation [35].

Viljanen *et al.* [39] concluded that IgA levels tended to be higher in probiotic groups than in placebo groups. Hence, a 4-week treatment with *Lactobacillus* GG may alleviate intestinal inflammation in infants with atopic eczema/dermatitis syndrome (AEDS) and cow's milk allergy (CMA). Monhan *et al.* [40] recorded that fecal IgA was higher in the probiotic group compared with the placebo group (p=0.021). The humoral immune response against *Salmonella* (serum IgM and IgA levels) was significantly greater in the probiotic group piglets than in control animals, suggesting that *E. faecium* NCIMB 10415 treatment enhanced the course of infection in weaning piglets challenged with *Salmonella* serovar Typhimurium DT 104[41]. However, the probiotic treatment also appeared to result in greater production of antibodies against *Salmonella* serovar Typhimurium.

In the present investigation, lysozyme activity was studied through the protected groups. The lysozyme had significantly increased on the second day after oral challenge and administration of La-5, Bb-12 and Lh-02 (Groups 2, 3, & 4) as shown in Table 8 and Figure 8. In the treated groups, the level of lysozyme was increased after 10 days of oral challenge with *Salmonella typhimurium* and treatment with ABT-3 and La-5 (Groups 6 and 7) compared to the level of lysozyme in

control group (G 8), as shown in Table 9 and Figure 9. Namba *et al.* [42] showed that lysozyme or digested cell walls presented by the oral route enhance the immune response in guinea pigs.

As shown in Table 10, the level of serum AST and ALT is significantly decreased among the protected and treated mice (G 1, 2, 3, 4, 6, & 7) compared to the control groups (G 5 & 8). Sayed [43] reported that kids supplemented with probiotics had significant increase in hemoglobin concentration, PCV %, erythrocyte count and blood serum total protein, while total leukocyte count, blood serum AST, serum urea and serum creatinine levels were not significantly altered. Antunovic *et al*. [44] recorded that the probiotic pioneer PDFM significantly reduced serum glucose and urea levels and activities of AST, ALT and CK but significantly increased the levels of total bilirubin and triglycerides in lambs.

The activities of AST and ALT in mice of the control negative group and probiotic supplemented groups in the experiment were in harmony with that detected by Sadiek and Bohm [45,46], who demonstrated that the activities of AST and ALT were normally and nearly the same in control and probiotic-treated animals, thus indicating that probiotic had no side effects on the animal health. Concerning liver health, the main benefits of probiotics might occur through preventing the production and/or uptake of lipopolysaccharides in the gut and therefore reducing levels of low-grade inflammation [35]. Hepatic fat metabolism also seems to be influenced by the presence of commensal bacteria and potentially by probiotics. This might be of major importance in the future because low-grade inflammation, hepatic fat infiltration and hepatitis might become more prevalent as a result of high fat intake and the increased prevalence of obesity [35].

Table 10 concluded that both the levels of creatinine and uric acid decreased significantly in the mice serum among protected or treated mice (G1, 2, 3, 4, 6, & 7) compared to control groups (G 5 & 8). Bakr *et al.* [45] concluded that serum creatinine and uric acid levels in the animals of control and probiotic treated groups were fluctuating and within the normal physiological ranges recorded by Benjamin [47]. No significance differences were recorded among the animal groups along the period of the experiment.

According to the results of Table 10, it is clear that probiotic protected or treated mice groups (G1, 2, 3, 4, 6, & 7) had significant antioxidant activity compared to the control groups (G 5 & 8). *Lactobacillus casei* or *Lactobacillus gasseri* expressing a manganese superoxide dismutase (MnSOD) can reduce inflammation via the inhibition of neutrophil recruitment [48,49]. It was previously shown that pharmacological inhibition of either NO or superoxide production resulted in a remarkable enhancement of *Salmonella* growth and increased mortality in murine salmonellosis, suggesting that both NO and superoxide contribute critically to the host defense against serovar Typhimurium [50]. It has been shown that some lactobacilli possess antioxidative activity and are able to decrease the risk of the accumulation of ROS during the ingestion of food [51,52]. Lactic acid bacteria are able to degrade the superoxide anion and hydrogen peroxide [53,54].

In conclusion, this study refers to the probiotics which have obvious curing effect on salmonellosis without any deleterious effect on animal health even when given in high doses. Also, it was found that using the mixed probiotic strains culture could increase the protective and treating effects against *Salmonella typhimurium* infection and is more effective than using the individual probiotic strain.

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